Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using avian model

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Hsiu-Lien Herbie Lin, Pascal Mermillod, Isabelle Grasseau, Jean-Pierre Brillard, Nadine Gérard, et al.. Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using avian model. Animal Reproduction Science, 2023, 258, pp.107330. 10.1016/j.anireprosci.2023.107330 . hal-04230575v2

HAL Id: hal-04230575
https://hal.inrae.fr/hal-04230575v2

Submitted on 10 Oct 2023

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1 **Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using**

2 **avian model**

3

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12

13 **Short title:** glycerol toxic effects on sperm fertilization

14 **Keywords:** cryoprotectant, fertility, glycerol, sperm, sperm storage tubules (SST), toxicity

15 **Word count:** 5000 words, excluding abstract, references and figure legends

16 **In brief:** Glycerol is a commonly used cryoprotectants for sperm cryopreservation in many

17 species but shows cytotoxicity to decrease fertility. This study demonstrates that glycerol

18 affects sperm migration and storage in the oviducts as well as negative modifications of

19 sperm biology.

20

ABSTRACT

Glycerol is a cryoprotectant used worldwide for sperm cryopreservation in animals but it is associated with decreasing fertility. The mechanism underlying glycerol effects remains unclear, thus here we aimed to better understand by using the chicken model. First, we checked the effect of increasing glycerol concentration at insemination on hen fertility, showing that 2 and 6% glycerol induced partial and total infertility. Subsequently, we examined sperm storage tubules (SST) colonizing ability during *in vivo* insemination and *in vitro* incubation of Hoechst stained sperm containing 0, 2 or 6% glycerol. Furthermore, we conducted perivitelline membrane lysis tests and investigated sperm motility, mitochondrial function, ATP concentration, membrane integrity and apoptosis, after 60 min of incubation with different glycerol concentrations (0,1, 2, 6 and 11%), at two temperatures to mimic pre-freezing (4°C) and post-insemination (41°C) conditions. Whereas 2% glycerol significantly reduced 50% of SST containing sperm, 6% glycerol totally inhibited SST colonization *in vivo*. On the other hand, *in vitro* incubation of sperm with SST revealed no effect of 2% glycerol and 6% glycerol showed only a 17% reduction of sperm filled SST. Moreover, glycerol reduced sperm-egg penetration rates as well as affected sperm motility, bioenergetic metabolism and cell death at 4°C when its concentration exceeded 6% and caused greater damages at 41°C, especially decreasing sperm motility. These data altogether reveal important effects of glycerol on sperm biology, sperm migration, SST colonization, and oocyte penetration, suggesting at least a part of fertility reduction by glycerol and open the way for improving sperm cryopreservation.

Key words: cryoprotectant, fertility, glycerol, sperm, sperm storage tubules (SST), toxicity

45 **Introduction**

46 Sperm cryopreservation is a practical strategy to struggle the biodiversity crisis of wild
47 animals (Bolton *et al.* 2022), to exchange superior genotypes in domestic animals (Bebbere
48 & Succu 2022), and to preserve fertility in humans (Tao *et al.* 2020). The cryopreservation
49 involves successive steps: semen collection, cryoprotectant addition, cellular dehydration,
50 freezing, storage in liquid nitrogen, and thawing prior to sperm use (Gupta *et al.* 2018; Ugur
51 *et al.* 2019). Cryoprotectants are chemical compounds that protect cells from cryodamages.
52 Among them, glycerol, an intracellular cryoprotectant, is the most commonly used one for
53 sperm cells in most species (Villaverde *et al.* 2013; Papa *et al.* 2015; Whaley *et al.* 2021; Ma
54 *et al.* 2022). However, it is associated with undesirable cytotoxic effects on sperm (Holt
55 2000; Morrier *et al.* 2002; Abouelezz *et al.* 2017), affecting sperm biological characteristics
56 and fertility (Fahy 2010; Best 2015) in various species including humans (McLaughlin *et al.*
57 1992), mice (Katkov *et al.* 1998), donkeys, horses (Demick *et al.* 1976; Vidament *et al.* 2009),
58 pigs (Wilmut & Polge 1974; Gutiérrez-Pérez *et al.* 2009), sheep (Slavik 1987; Abdelhakeam *et*
59 *al.* 1991) and marsupials (Taggart *et al.* 1996; Rodger *et al.* 2009).

60 To avoid its cytotoxic effects, different strategies were developed such as mixing
61 glycerol with other cryoprotectants during cryopreservation process (Yildiz *et al.* 2007; Silva
62 *et al.* 2012; Blanch *et al.* 2014) or its removal before insemination (Seigneurin & Blesbois
63 1995; Purdy *et al.* 2009). While these methods can partly alleviate its negative effects, little
64 is known about the mechanisms involved in glycerol toxicity, directly on sperm biology
65 and/or through modifications of the female genital tract.

66 In chickens, glycerol present in semen may dramatically induce total infertility with
67 only half of the concentration used for cryopreservation (Polge 1951; Neville *et al.* 1971).
68 During the production and use of chicken cryopreserved semen, sperm face different

69 temperatures: 4°C during the glycerol addition, equilibration, decreasing to -196°C during
70 freezing and storage, 4°C again after thawing, and finally 41°C after insemination into the
71 female reproductive tract (Lin *et al.* 2022). While this cryopreservation method is quite
72 efficient, the combination of glycerol presence at 4°C and 41°C may have direct effects on
73 sperm biology in addition to their journey inside the oviduct, glycerol may impair sperm
74 migration and storage into the sperm storage tubules (SST), a specific well developed organ
75 involved sperm selection, survival, and maintenance of fertilizing capacity, similar to the
76 sperm reservoir of mammalian fallopian tubes (Camara Pirez *et al.* 2020; Mahé *et al.* 2021).
77 The deficit of sperm storage in SST and its release to reach the oocyte in the oviduct may
78 impair final fertility (Machado *et al.* 2019; Kölle 2022). Furthermore, glycerol toxic effects
79 may also reduce sperm ability to penetrate the coverage of the oocyte, known as the inner
80 perivitelline membrane (IPVM) in birds, analogous to the mammalian zona pellucida (ZP)
81 (Ichikawa *et al.* 2017), leading to a fertilization failure.
82 Consequently, using the avian model, here we aimed to decipher the effects of glycerol
83 on sperm fertilizing ability, including direct effects on sperm biology as well as the
84 interaction of sperm with female genital tract. We firstly determined the critical
85 concentrations of glycerol in semen associated with chicken fertility reduction by
86 insemination tests. Subsequently, we explored the sperm capacity to reach and be stored in
87 the oviduct with both *in vivo* and *in vitro* SST colonization experiments. Then, we
88 investigated sperm ability to penetrate perivitelline membrane *in vitro*. Finally, we evaluated
89 the direct effects of glycerol on sperm biology during pre-freezing (4°C) and post-
90 insemination (41°C) temperature conditions, on sperm motility, mitochondrial activity, ATP
91 generation, membrane integrity and apoptosis.
92

93

94 **Materials and methods**

95 **Animal management**

96 All experiments were conducted in accordance with the legislation governing animal
97 treatment and were approved by the French Ministry of Higher Education, Research and
98 Innovation, and the Val-de-Loire Animal Ethics Committee (authorization number: N°
99 APAFIS#4026-2016021015509521 and APAFIS#34415-202112141205965). A total of 20 adult
100 Sasso T-44 roosters were randomly allocated to 2 groups for fertility tests and sperm biology
101 parameters study (motility, mitochondrial activity, ATP concentration, viability, sperm-SST
102 storage and PVM penetration). Two herds of 60 adult Lohmann hens were housed by groups
103 of 4, one for fertility tests and the other for SST and PVM experiments. All animals were
104 under a lighting regimen of 14h light:10h dark, controlled temperature at 20°C, feeding with
105 a standard diet and water *ad libitum* at the INRAE Poultry Experimental unit
106 (https://doi.org/10.15454/1.5572326250887292E12). Animals were between 35 and 85-
107 week-old during this study.

108

109 **Semen collection and processing**

110 Rooster semen was collected in a tube containing 200 µL of Lake PC diluent (Lake &
111 Ravie 1981) by abdominal massage (Burrows & Quinn 1937). Semen samples were then
112 pooled and diluted to 2 000 × 10^6 cells/mL with Lake PC diluent at room temperature for the
113 following treatments. The number of all experimental replicates are given in the Figure
114 legends.

115

116 **Fertility tests**

117 After collection, pooling and adjustment of glycerol concentration to 0, 1, 2, 6 and 11%
118 (v/v), semen was immediately used for intravaginal insemination (4 cm depth), with a dose
119 of 100×10^6 sperm/hen for 2 consecutive days (12 hens/experimental group). Eggs were
120 collected from day 2-7 after first insemination and stored at 15°C/85% humidity before
121 incubation at 37.7°C/55% humidity. Fertile and infertile eggs were determined by candling at
122 the 7th day of incubation (Long & Kulkarni 2004). Repeated manipulation with exchanged
123 group of hens used to confirm the previously observed results (Supplementary Data 1).
124
125 **Sperm- SST test**
126 **Sperm staining**
127 Sperm was labeled with the previous methodologies (McDaniel *et al.* 1997; King *et al.*
128 2002). After collection, pooling and dilution, 40 µL of 1 mg Hoechst 33342 mL^{-1} (Sigma-
129 Aldrich) was added to 1 mL of diluted semen and gently mixed on an orbital shaker at 4°C for
130 4 h. The fertilizing ability of stained sperm is given in Supplementary Data 2, showing that
131 Hoechst labeling did not affect fertility tendency.
132 ***In vivo* insemination**
133 Hoechst stained sperm were divided to 3 groups and added glycerol to a final
134 concentration of 0 (control), 2 and 6% of glycerol. A total of 3 hens per group were
135 inseminated twice at 24 h intervals with a dose of 200×10^6 sperm/female (King *et al.* 2002)
136 then slaughtered 24 h after the second insemination and oviducts were isolated. The villi
137 (n=6 per animal) containing SST were separated randomly from uterovaginal mucosa
138 (Cordeiro *et al.* 2018). Villi pieces were fixed in 4% paraformaldehyde solution at 37°C for 30
139 min then mounted in Fluoromount-G™ medium (ThermoFisher) on microscope slides.
140 Images were acquired using a microscope slide scanner (Axio Scan.Z1, Zeiss) with an

141 objective lens 20X. Fluorescence imaging was performed with X-Cite Illumination System and
142 emission Band Pass of EM BP 445/50 (DAPI) and EM BP 690/50 (Alexa Fluor 633) used
143 respectively for Hoechst labeled sperm and SST autofluorescence. The presences of sperm-
144 filled or sperm-empty SST were identified manually with QuPath image analysis software
145 and the percentage of SST filled with stained sperm was calculated.

146 ***In vitro* incubation**

147 For this experiment, sperm staining and the uterovaginal villi collection were
148 performed as previously described. Hoechst labeled sperm were washed in Lake PC diluent
149 to remove extra staining solution and centrifuged at 600×g for 20 min at 4°C. Sperm pellets
150 were resuspended and adjusted with Lake PC diluent to 2 000 × 10^6 cells/mL then mixed
151 with glycerol to final concentration of 0 (control), 2 and 6% glycerol. For each treatment, 6
152 pieces of villi were placed individually in 1 mL of Advanced DMEM/F-12 medium
153 (ThermoFisher, Gibco#12634-010) containing 0, 2 and 6% of glycerol in a 4-well culture dish.
154 Each well was inseminated with 200 × 10^6 sperm and incubated at 39°C for 30 min in 5% CO_2
155 and 95% humidity. After incubation, villi pieces were washed in Dulbecco's phosphate-
156 buffered saline (DPBS) to remove sperm adhering outside the SST, then fixed and analyzed
157 as previously described.

158

159 **Sperm-PVM perforation test**

160 **Isolation of perivitelline membrane (PVM)**

161 PVM was isolated according to the method described previously (Steele *et al.* 1994;
162 Bongalhardo *et al.* 2009). Yolk membrane was separated from freshly unfertilized chicken
163 eggs and washed several times in DPBS to remove adherent yolk. Segments excluding

164 germinal disc area of PVM were cut in the size of 75 mm × 75 mm and placed in Advanced
165 DMEM/F-12 medium before use.

166 **Sperm glycerolized treatment**

167 After collection and pooling, semen was diluted with glycerol-Lake PC diluent to 200 ×
168 10^6 sperm cells/mL and final concentration of 0 (control), 1, 2, 6 and 11% glycerol then
169 incubated at 4 or 41°C for 10 min.

170 ***In vitro* incubation of PVM and glycerolized sperm**

171 A piece of PVM placed in 1 mL of Advanced DMEM/F-12 medium was incubated with
172 10 × 10^6 sperm of each condition (0, 1, 2, 6 and 11%) in a 24-well culture dish at 41°C for 20
173 min. After incubation, PVM segments were washed in DPBS and fixed in 4%
174 paraformaldehyde solution at 37°C for 1 min (Ichikawa *et al.* 2017). The PVM sections were
175 then mounted on microscope slides, stained with Schiff's reagent (Sigma-Aldrich) and air-
176 dried (Akhlaghi *et al.* 2014). The images of holes that formed in the PVM were captured by
177 the microscope slide scanner with brightfield optics of an objective lens 20X. Three regions
178 were randomly selected from each PVM and the number of holes was counted manually
179 with QuPath software to calculate the number of holes/mm^2.

180

181 **Evaluation of sperm quality parameters**

182 After semen collection and dilution, glycerol was added to reach final concentration of 0
183 (control), 1, 2, 6 and 11% and then incubated at 4 or 41°C for 60 min.

184 **Sperm motility**

185 Sperm motility were examined with a computer-assisted sperm analysis system (CASA,
186 IVOS, IMV Technologies) as previously described (Vitorino Carvalho *et al.* 2021). The
187 evaluation was performed with a concentration of 30 × 10^6 sperm/mL. Based on several

188 motility parameters, i.e. average path velocity (VAP), straight line velocity (VSL) and
189 straightness (STR = VSL/VAP), motility results were indicated as percentage of motile sperm
190 and progressive sperm, which were defined as the percentage of sperm showing a VAP > 5
191 µm/sec and VAP > 50 µm/sec with STR > 75%, respectively.

192 **Mitochondrial activity**

193 Sperm mitochondrial parameter was evaluated with a fluorescent probe JC-1 (Sigma-
194 Aldrich, CAS NO.: 3520-43-2), a green-fluorescent monomer at low membrane potential and
195 forms red-fluorescent aggregates at higher potential (Gliozzi *et al.* 2017). Semen was diluted
196 to 1×10^7 sperm/mL in PBS to final volume of 200 µL and then incubated with 1 µg/mL of JC-
197 1 dye at 37°C for 30 min. A total of 5 000 events of each sample were analyzed by Guava®
198 easyCyte (IMV Technologies). The results were expressed as the percentage of sperm with
199 mitochondrial membrane depolarization.

200 **ATP concentration**

201 A luciferase reaction assay (CellTiter-Glo® Luminescent Assay Kit, Promega#G7570)
202 was used to measure sperm ATP concentration here (Nguyen *et al.* 2015). After incubation
203 with glycerol, semen samples were immediately centrifuged at 4°C, 800×*g* for 10 min,
204 supernatant was removed and the pellets were frozen at -20°C. Before ATP assay, sperm
205 pellets were thawed and resuspended in 100 µL PBS in the 96-well white polystyrene
206 microplate. After 30 min of equilibration at room temperature, 100 µL luciferin/luciferase
207 reagent was added to each well and plates were placed on an orbital shaker for 2 min to
208 induce cell lysis then waited for 10 min to stabilize the luminescent signal. The luminescence
209 was recorded with the luminometer plate reader (CLARIOstar, BMG LABTECH) and
210 transformed to ATP concentration based on a standard curve.

211 **Membrane breakage**

212 This parameter was explored by SYBR 14/Propidium iodide (PI) double fluorescent
213 staining technique (Molecular Probes, Invitrogen #L7011) combined with a flow cytometer
214 Guava® easyCyte (Gliozzi *et al.* 2017). PI positive sperm (red fluorescence) were recognized
215 as membrane damaged sperm and SYBR 14 ones (green fluorescence) were considered as
216 intact sperm. The results were expressed as percentage of sperm showing membrane
217 breakage.

218 **Apoptosis**

219 Annexin V-binding technique (Hoogendijk *et al.* 2009) was used to determine sperm
220 apoptotic cells according to the manufacturer's instructions (Novus Biologicals™#NBP2-
221 29373). Briefly, 1.5 µL semen was washed in 1 mL of PBS twice. Sperm pellets were obtained
222 by centrifuge at 400×*g* for 5 min and resuspended in 55 µL of Annexin V-FITC and PI mix
223 staining buffer. After 20 min of incubation in the dark, 200 µL of assay buffer were added
224 and samples were analyzed by Guava® easyCyte within 1 h.
225

226 **Statistical Analysis**

227 Statistical analyses were performed with GraphPad Prism version 6.07. The impact of
228 glycerol concentration on fertility was analyzed by Chi-square test and Fisher's exact test.
229 Percentage of SST filled with sperm were analyzed by Kruskal-Wallis and Dunn's multiple
230 comparisons tests to evaluate the effect of glycerol concentration. Significant differences
231 were considered when p-value was under 5%. Sperm-PVM penetration data were analyzed
232 by two-way ANOVA test to examine the effects of glycerol concentration and exposure
233 temperature. All sperm quality parameters results were analyzed by two-way ANOVA test to
234 examine the effects of glycerol concentration and exposure time. Since exposure time had
235 no effect on all sperm quality parameters at 4°C (Table 1), data from different timepoints

236 were pooled in a mixed model to highlight the effects of glycerol concentration. Differences
237 in the means were analyzed by Tukey's HSD Post Hoc test.
238
239
240 **Results**
241 **Impact on animal fertility**
242 Whereas 1% glycerol had no effect on the number of fertilized eggs (Figure 1), the
243 presence of 2% glycerol significantly reduced the fertility by about 50% while 6% and 11% of
244 glycerol resulted in total infertility. Same effect was reproduced in the second replicate test
245 (Supplementary Data 1).
246
247 **Impact on sperm presence in SST**
248 The concentrations of 0, 2 and 6% glycerol were chosen in SST experiments based on
249 the fertility results. Indeed, the presence of 2 and 6% glycerol in semen led to a partial and
250 total loss of fertility, respectively, compared to 0%. We firstly confirmed that this effect was
251 the same after Hoechst staining protocol (Supplementary Data 2). Subsequently, the
252 capacity of sperm to reach and be stored in SST was investigated by inseminating Hoechst
253 stained sperm then SST dissection (Figure 2A). The percentage of sperm filled SST
254 significantly decreased as the glycerol concentration increased (Figure 2B): 2% and 6%
255 glycerolized sperm dramatically reduced the percentage of SST containing sperm
256 respectively by 50% and by nearly 100%.
257 To focus our work on the SST capacity to host sperm (avoiding possible motility effects
258 preventing sperm to reach SST), same approach was applied with *in vitro* incubation of
259 uterovaginal villi and glycerolized sperm (Figure 3A). No effect was observed with 2%

260 glycerol (Figure 3B), while 6% glycerol significantly decreased 17% the proportion of SST
261 colonized by stained sperm.
262
263 **Effect on sperm-PVM penetration**
264 In order to evaluate the effect on fertilizing capacity, sperm was incubated 10 min with
265 different glycerol concentration at 4 and 41 °C and the number of holes generated in PVM by
266 sperm hydrolysis (Figure 4A) was analyzed. Whereas no interaction between glycerol
267 concentration and the temperature was detected, each variable independently affected the
268 number of holes in PVM (Figure 4B), showing less holes with increasing glycerol
269 concentrations. While a higher reduction of fertilizing ability was observed with the
270 incubation at 41°C than at 4°C (Supplementary Data 3), no significant difference was found
271 by pair-wise comparisons.
272
273 **Effect on sperm biology with an incubation at 4°C**
274 In order to collect new data relating to sperm biology affected at the specific stages
275 during the pre-freezing procedure, several aspects of sperm quality parameters were
276 evaluated including sperm motility, mitochondrial function, ATP concentration, membrane
277 integrity and apoptosis in presence of various concentrations of glycerol within 1 hour of
278 incubation at 4°C. At this temperature, no effect of exposure time and its interaction with
279 glycerol concentration was observed on all evaluated parameters (Table 1). Consequently,
280 we further combined all timepoints to highlight only the effects of glycerol concentration
281 (Supplementary Data 4). When compared to 0% glycerol, no effect of 1 and 2% glycerol was
282 observed on all sperm biological characteristics (Figure 5A-F), except that 2% glycerol
283 significantly increased ATP concentration (Figure 5D). Furthermore, 6% and 11% glycerol

284 decreased sperm motility and increased the values of all other sperm parameters when
285 compared to control condition. Moreover, 11% glycerol caused more pronounced negative
286 influence than 6%, except on mitochondrial function (Figure 5).
287
288 **Effect on sperm biology with an incubation at 41°C**
289 We then explored the same sperm biology functions after glycerol exposure at 41°C, to
290 mimic the impact of temperature after sperm insemination into female reproductive tract,
291 within 1 hour (the physiological time necessary for sperm to reach the fertilization site in the
292 oviduct). Based on our preliminary results, sperm motility traits were observed within 30
293 min of incubation time due to their fast alterations when exposed to glycerol at 41°C. The
294 tendencies of percentage of motile and progressive sperm were very similar and were both
295 significantly affected by glycerol concentration, exposure time and their interaction (Table
296 1). Indeed, whereas no impact of 1, 2 and 6% of glycerol was revealed at 0 min, the addition
297 of 11% glycerol immediately dropped sperm motility (Figure 6). A similar negative effect was
298 also induced by 6% glycerol after 10 min of exposure (Figure 6). In addition to the reduction
299 of sperm motility classically induced by the incubation of chicken sperm at 41°C (decreasing
300 pattern observed with control condition over time), at 20 and 30 min, all glycerol
301 concentrations reduced drastically sperm motility in a dose dependent manner, leading to
302 sperm nearly immobile with 11% of glycerol at 20 min.
303 Significant effects of glycerol concentration, exposure time and their interaction were
304 observed on mitochondrial function (Table 1). Whereas at 0 min, no difference was observed
305 in all glycerol concentrations, a significant increase of mitochondria depolarization was
306 observed at 30 min in absence of glycerol when compared to 0 min. Interestingly,
307 mitochondria depolarization was higher in 60 min than all other timepoints and it

308 significantly decreased with the increase of glycerol concertation (Figure 7A). Glycerol
309 concentration, exposure time and their interaction also significantly affected energy
310 metabolism as revealed by sperm ATP concentration evaluation (Table 1). Indeed, whereas
311 no significant influence was observed for 1 and 2% glycerol conditions at all timepoints
312 (Figure 7B), an important increase of ATP concentration was revealed with 11% of glycerol at
313 30 min as well as 6% of glycerol at 60 min, when compared to control, and to 1 and 2%
314 glycerol conditions. At 60min, the ATP concentration observed with 11% was higher than the
315 one obtained with 6% of glycerol.

316 In order to clarify the effect of glycerol on sperm cell survival, two parameters were
317 evaluated: the presence of sperm membrane breakage and sperm apoptosis. Both
318 parameters presented very closed patterns with a significant increase of their level due to
319 glycerol concentration and exposure time, with no significant interaction (Table 1). At 0 min,
320 no impact was observed on the membrane breakage rate whereas an increase in apoptotic
321 cell percentage was found immediately after the addition of 11% glycerol. At 30 and 60 min,
322 the rates of membrane breakage and apoptotic sperm cells significantly increased only for 6
323 and 11% when compared to the other glycerol concentrations as well as to these
324 experimental conditions at 0 min (Figure 8).

325

326

327 **Discussion**

328 Whereas glycerol is one of the most widely used cryoprotectant to preserve sperm at
329 very low temperature in animals, its presence is also associated to undesired negative
330 effects on fertility (Macías García *et al.* 2012). In chickens, this negative influence could lead
331 to total infertility depending on the glycerol concentration present in inseminated semen

332 (Polge 1951; Seigneurin & Blesbois 1995). However, the cellular and molecular mechanisms
333 underlying the balance between maintaining sperm integrity and inducing toxic effects when
334 glycerol acts as a cryoprotectant remain unclear.

335 Here, using insemination program with chicken semen combined with different
336 glycerol concentrations, we determined that the presence of 2% glycerol harmfully reduced
337 animal fertility to half (91 to 43%) and that 6% glycerol induced total infertility (Figure 1 and
338 Supplementary Data 1). These results are consistent with previous studies performed with
339 different insemination programs and animal genotypes (Polge 1951; Neville *et al.* 1971),
340 suggesting that glycerol toxic effects appear regardless of animal genetic background, age or
341 insemination protocols in this species. Whereas 7% glycerol has no effect on pregnancy rates
342 in cattle (Papa *et al.* 2015), the addition of only 2.2% glycerol in donkey semen induces
343 infertility (Vidament *et al.* 2009). Consequently, although a cytotoxic glycerol effect could be
344 presumed for all animal species, some important variability of sensitivity can be observed.
345 However, very little information is available concerning about the stage and target of this
346 toxicity, i.e. direct impacts on sperm biology or/and effects on the events occurring inside
347 the female tract.

348 Once semen is inseminated into hen vagina, a population of sperm first reaches the
349 SST to be stored before continuing their journey to fertilize the oocyte in the oviduct. Our
350 data revealed that semen containing 2% glycerol showed a distinguishable decreased ability
351 to colonize the SST and that 6% glycerol led to sperm absence in SST (Figure 2). This
352 diminution or absence of sperm in SST, already mentioned previously (Marquez &
353 Ogasawara 1977), is certainly one of the major causes of fertility reduction induced by the
354 glycerol presence (Supplementary Data 2). However, this experiment did not discriminate
355 that sperm absence in SST was due to the failure of sperm migration (from vagina to SST) or

356 of entering and binding into SST. To answer this question, the *in vitro* experiment was
357 designed to exclude the defect of sperm migration from vagina to UVJ to see if glycerolized
358 sperm can be found in SST if they are deposited close from SST. Different from *in vivo*
359 experiment, sperm were present in SST after *in vitro* incubation of sperm with UVJ villi.
360 Intriguingly found that no impact of 2% glycerol on the rate of sperm filling in SST (Figure 3)
361 and just a partial reduction of 17% with 6% glycerol, a relatively slight loss when compared
362 to *in vivo* result (100% reduction), suggesting that small amount of glycerol impairs sperm
363 travel from vagina to SST *in vivo*, and that higher quantity of glycerol also inhibits sperm
364 depositing into SST. These negative effects may be due to motility defect and/or sperm
365 rejected by vagina selection (Brillard 1993; Bakst *et al.* 1994) as well as another barrier at the
366 SST level acting as a gatekeeper (Brady *et al.* 2022) to exclude glycerolized sperm.
367 After 30 min *in vitro* incubation of sperm with UVJ villi, we found that 6% glycerol did
368 not impair sperm motility (Supplementary Data 5), showing a completely different result
369 from another sperm motility observation. In that experiment, sperm were incubated with
370 with 6% glycerol without UVJ villi, leading sperm to lose almost their entire motility within
371 30 min (Figure 6). This finding may suggest that UVJ tissues provide a positive effect on
372 motility maintenance, which has been proposed in a previous study (Spreen *et al.* 1990).
373 In addition to affect sperm transport and storage, glycerol may also directly influence
374 sperm fertilizing ability including its potentiality to release proteolytic enzymes to hydrolyze
375 the perivitelline membrane (PVM), a crucial step to penetrate and fertilized the oocyte
376 (Lemoine *et al.* 2011; Priyadarshana *et al.* 2020). The higher the glycerol concentration was,
377 the lesser the sperm hydrolysis activity was (Figure 4), proving that glycerol impairs chicken
378 sperm fertilizing capacity and, this effect was more pronounced at 41°C than at 4°C
379 (Supplementary Data 3), suggesting a synergic effect between temperature and glycerol

380 concentration. To our best knowledge, these data are the first revealing glycerol effects
381 directly on sperm-egg penetration in birds. However, a different observation was reported in
382 rams, indicating that glycerol accelerates egg penetration and polyspermy (Slavik 1987),
383 suggesting a species specific effect remaining to be confirmed.

384 The sperm processing for freezing, thawing and insemination involves mainly two
385 temperatures: low temperature (4°C) used during sperm preparation and physiological body
386 temperature (41°C) after insemination into the female tract. These temperatures were
387 considered here to decipher the direct impacts of glycerol on sperm biology to better
388 understand which stages of the process the sperm are affected. At 4°C, despite the presence
389 of less than 2% of glycerol had no effect on sperm biology within 60 min, more than 6% of
390 glycerol significant decreased sperm motility and viability, and increased mitochondrial
391 activity and ATP concentration (Figure 5), revealing that the modification for current freezing
392 protocol might be useful to reduce undesired glycerol effects since the most used glycerol
393 concentration for chicken sperm cryopreservation is more than 8% (Mocé *et al.* 2010; Zong
394 *et al.* 2022). Decreasing the amount of glycerol to less than 2% may be a possible solution by
395 replacing its requirement with other cryoprotectants. However, cytotoxic effects of other
396 cryoprotectants, such as DMA and DMSO, cannot be ignored (Best 2015; Mosca *et al.* 2019).
397 An option would be to mix several cryoprotectants to have a sufficient cryoprotection while
398 maintaining each individual under its own toxicity threshold. Designing a satisfactory and
399 harmless sperm cryopreserved method remains an endeavor and a challenge even after 70
400 years of development. Another finding in our study was the absence of effect of exposure
401 time on sperm biology at 4°C within 1 h (Table 1 and Figure 5). This information could be
402 very useful to add more flexibility to insemination programs, by keeping semen at 4°C

403 maximum 1 hour in farm environment. However, *in vivo* data on animal fertility are still
404 needed to verify this potential procedure modifications.
405 At 41°C (physiological body temperature), the situation is dramatically different.
406 Indeed, sperm motility was rapidly decreased by glycerol in a concentration dependent way,
407 leading to a strong motility reduction more than 90% within 20 min in the presence of 11%
408 glycerol (Figure 6). Consequently, this severe disorder of sperm motility could disturb normal
409 sperm progression to reach SST or the fertilizing site in the oviduct since this whole journey
410 takes only 1 hour *in vivo* (Bakst *et al.* 1994). Short-term exposure of glycerol at room
411 temperature in 20 min also led to a significant reduction of sperm motility in humans
412 (McLaughlin *et al.* 1992) and mice (Katkov *et al.* 1998), suggesting that it's not a chicken
413 specific problem. At this temperature, higher concentration of glycerol (6 and 11%) also
414 increased sperm membrane breakage, a typical feature of necrosis, and apoptosis as
415 evidenced by Annexin V test (Figure 8). Whereas similar results were observed for
416 membrane breakage of stallion sperm under 5% glycerol condition, no effect was observed
417 on apoptotic rate (Macías García *et al.* 2012), suggesting a different sensitivity of glycerol
418 effects on sperm cell death patterns depending on the species. Alternatively, modifying a
419 glycerol freezing diluent for chicken sperm by adding apoptotic regulators such as
420 agonist/antagonist of death receptor signaling or caspase inhibitors (Fischer & Schulze-
421 Osthoff 2005) might be a possible way to limit glycerol cell death induction. Furthermore,
422 this increase of sperm death may also be one of the causes of the reduction of sperm
423 presence in SST. Since SST are known to select and maintain higher quality sperm, sperm
424 presenting death signals may be rejected (Brillard 1993; Blesbois & Brillard 2007).
425 Lastly, glycerol changes chicken sperm energy metabolism with a dramatic increase of
426 ATP concentration and a lower mitochondrial depolarization (Figure 7), corresponding to

427 previous studies in stallions (Macías García *et al.* 2012) and boars (Malcervelli *et al.* 2020).
428 During ATP synthesis via mitochondrial respiration, the displacement of protons generates a
429 voltage gradient to polarize the membrane with positive charge outside, defined as
430 mitochondrial membrane potential (Wang *et al.* 2022). Thus mitochondria depolarization,
431 refers to sperm with low mitochondrial membrane potential, generally associates with ATP
432 deficiency and lower sperm motility (Alamo *et al.* 2020). Here sperm incubated at 41°C for
433 30 min showed no difference of mitochondria depolarization in each glycerol condition
434 (Figure 7A), theoretically generating a comparable ATP content in each of them (Pandey *et*
435 *al.* 2021). However, glycerol concentrations of 6 and 11% led to a 25 and 60-fold higher ATP
436 concentrations than the other conditions (Figure 7B), raising the hypothesis that excessive
437 ATP is synthesized via glycolysis or pentose phosphate pathways (Setiawan *et al.* 2020).
438 However, further studies will be needed to demonstrate direct evidence of the activity of
439 these pathways.
440 Dynein, a specific ATPase uses energy from ATP hydrolysis and distributes it along the
441 axonemal microtubules, the central cytoskeletal structures of sperm flagellum, to generate
442 sperm movement (Sengupta *et al.* 2020). Theoretically, sperm with higher ATP contents
443 show higher motility (Alamo *et al.* 2020). However, here we observed that at 30 min ATP
444 accumulation in sperm under 6 and 11% glycerol conditions (Figure 7B) contrastingly
445 decreased their motility performance (Figure 6). This finding raises another hypothesis that
446 glycerol may disrupt dynein enzymatic activity or flagellar axonemal structure, leading to the
447 accumulation of unused ATP.
448 To conclude, we used the chicken model to demonstrate that glycerol affects severely
449 sperm capacity to interact with female reproductive tract, disturbing sperm migration and
450 storage (Figure 9) and reducing sperm ability to penetrate the ovum and achieve fertilization

in the oviduct. We also proved that these effects occur mainly at the physiological body temperature, suggesting a good sperm capacities preservation by the glycerol during cryopreservation/thawing process. Consequently, although understanding the entire mechanisms of glycerol toxicity remains a challenge, it is clear that removing glycerol prior to insemination is a valuable solution to avoid fertility reduction in chickens and should be also considered in mammalian species.

Declaration of interest

The authors declare that there are no conflicts of interest.

Funding

This study was performed with the financial support of CRB anim (ANR-11-INBS-0003) and COA, Taiwan (112AS-1.3.1-ST-m5).

Author contribution statement

PM, EB and AVC conceived the original idea. H-LHL and IG performed the experiments. H-LHL and AVC analyzed the data. J-PB, NG, KR and L-RC aided in interpreting the results. H-LHL, PM, and AVC wrote the manuscript. All authors discussed the results, commented on the manuscript and approved the submitted version.

Acknowledgments

We thank Maryse Meurisse and Marie-Claire Blache for their help with image analysis. We thank the staffs of the UE-PEAT, INRAE, Nouzilly, France for all assistance with animal care and experiments.

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686 the quality and fertility of frozen chicken semen. *Poultry Science* **101** 101840.
687 (10.1016/J.PSJ.2022.101840).
688
689

690 **TABLE**

691 **Table 1. Effect of glycerol concentration (GLY) and exposure time (T) on *in vitro* sperm**
692 **quality parameters.**

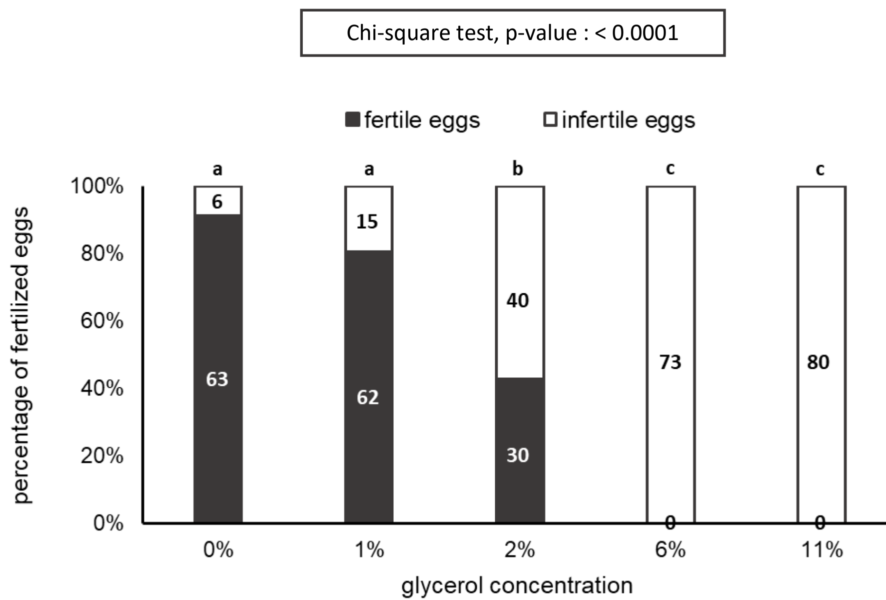
	two-way ANOVA test (p-value)					
	4°C			41°C		
parameter	GLY	T	GLY × T	GLY	T	GLY × T
motile sperm	**< 0.0001**	0.9103	0.8219	**< 0.0001**	**< 0.0001**	**< 0.0001**
progressive sperm	**< 0.0001**	0.9633	0.9839	**< 0.0001**	**< 0.0001**	**< 0.0001**
mitochondria activity	**0.0192**	0.0676	0.9894	**< 0.0001**	**< 0.0001**	**0.0004**
ATP concentration	**< 0.0001**	0.9520	0.9767	**< 0.0001**	**0.0049**	**< 0.0001**
membrane breakage	**0.0041**	0.4032	0.9609	**< 0.0001**	**< 0.0001**	0.3196
apoptosis	**< 0.0001**	0.0851	0.9361	**< 0.0001**	**< 0.0001**	0.5577

693 P-values lesser than 0.05 were considered as significant (in bold). Data of motile and progressive sperm
694 were observed at 0, 10, 20 and 30 min and the other parameters were observed at 0, 30 and 60 min.
695

696 **Figures**

697

698

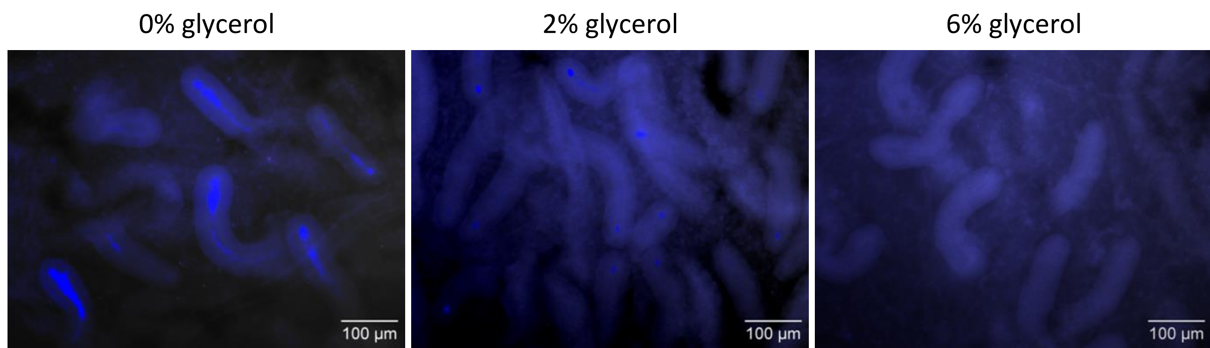


699

700 **Figure 1. Effect of glycerol presence in semen samples on sperm fertility capacity.** Semen
701 was collected and pooled from 10 roosters. Number of hens=12 for each experimental
702 condition. Eggs were collected between 2nd and 7th day after first insemination in a dose of
703 100×10^6 sperm per hen for 2 consecutive days. Black and white bars represent the
704 percentage of fertile and infertile eggs. The numbers of considered eggs are indicated on the
705 bars. Different letters indicate significant differences (p-value < 0.05).

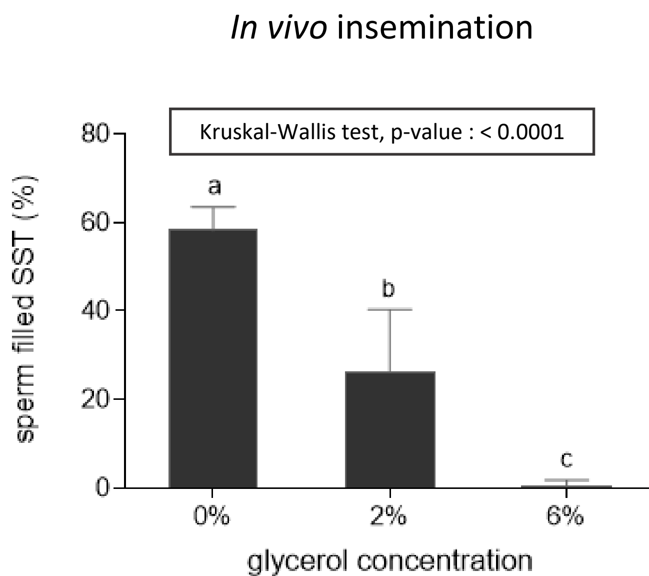
706

707 A



708

709 B

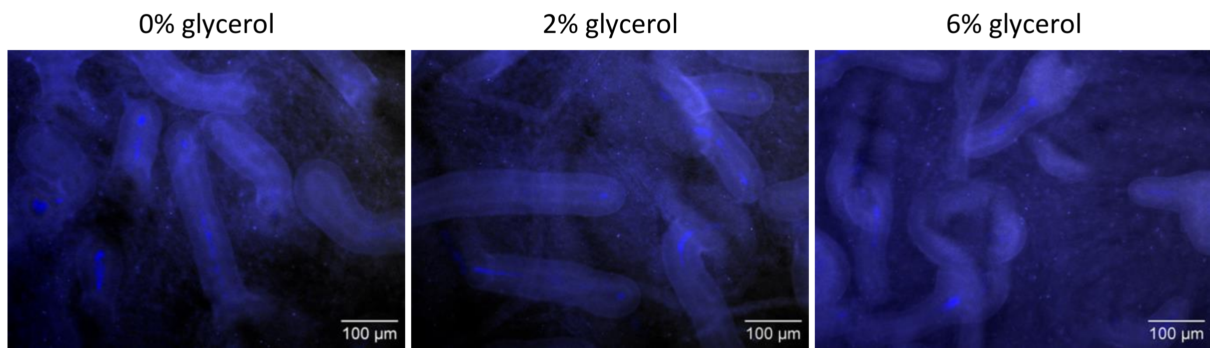


710

711 **Figure 2. The presence of sperm in sperm storage tubules (SST) after *in vivo* insemination.** A
712 and B represent sperm identification (A) and the percentage of SST containing sperm (B) after
713 *in vivo* insemination with 0, 2 and 6% glycerolized semen. Sperm stained with Hoechst 33342
714 at 4°C for 4 h then mixed with glycerol just before insemination. SST were detected by
715 autofluorescence. Data collected from 6 villi of each treatment. The bars and lines correspond
716 to the mean (3 experimental replicates) and the standard deviation of the mean. Different
717 letters indicate significant differences (p-value < 0.05).

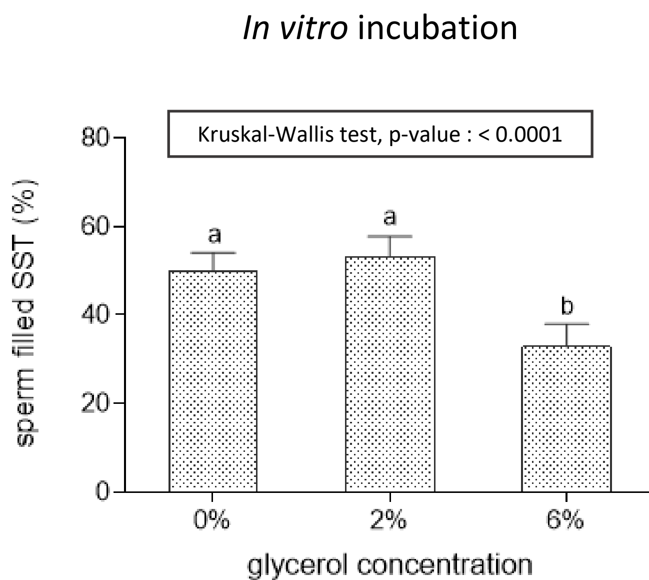
718

719 A



720

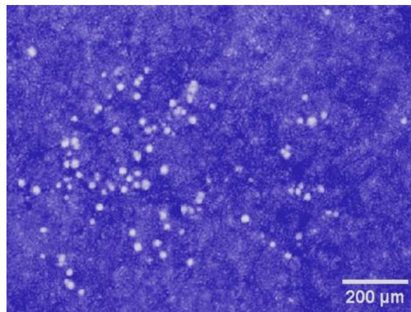
721 B



722

723 **Figure 3. The presence of sperm in sperm storage tubules (SST) after *in vitro* incubation.** A
724 and B represent sperm identification (A) and the percentage of SST containing sperm (B) after
725 *in vitro* incubation of sperm and uterovaginal villi in 0, 2 and 6% glycerol medium. Sperm
726 stained with Hoechst 33342 at 4°C for 4 h and washed in Lake PC diluent then mixed with
727 glycerol just before incubation. SST were detected by autofluorescence. Data collected from
728 3 villi of each treatment. The bars and lines correspond to the mean (5 experimental
729 replicates) and the standard deviation of the mean. Different letters indicate significant
730 differences (p-value < 0.05).
731

732 A

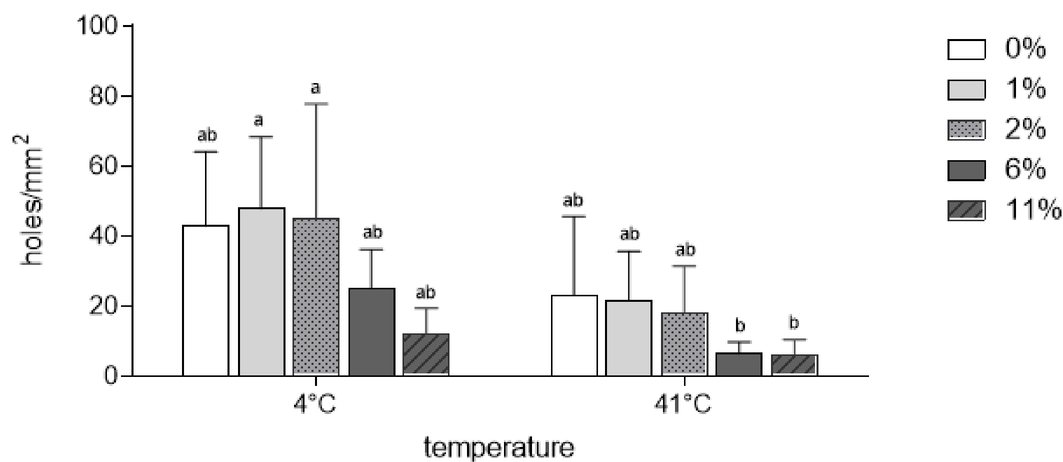
733 

734

735 B

two-way ANOVA test (p-value)			
	GLY	temperature	GLY × temperature
number of holes in PVM	**0.0044**	**0.0003**	0.6720

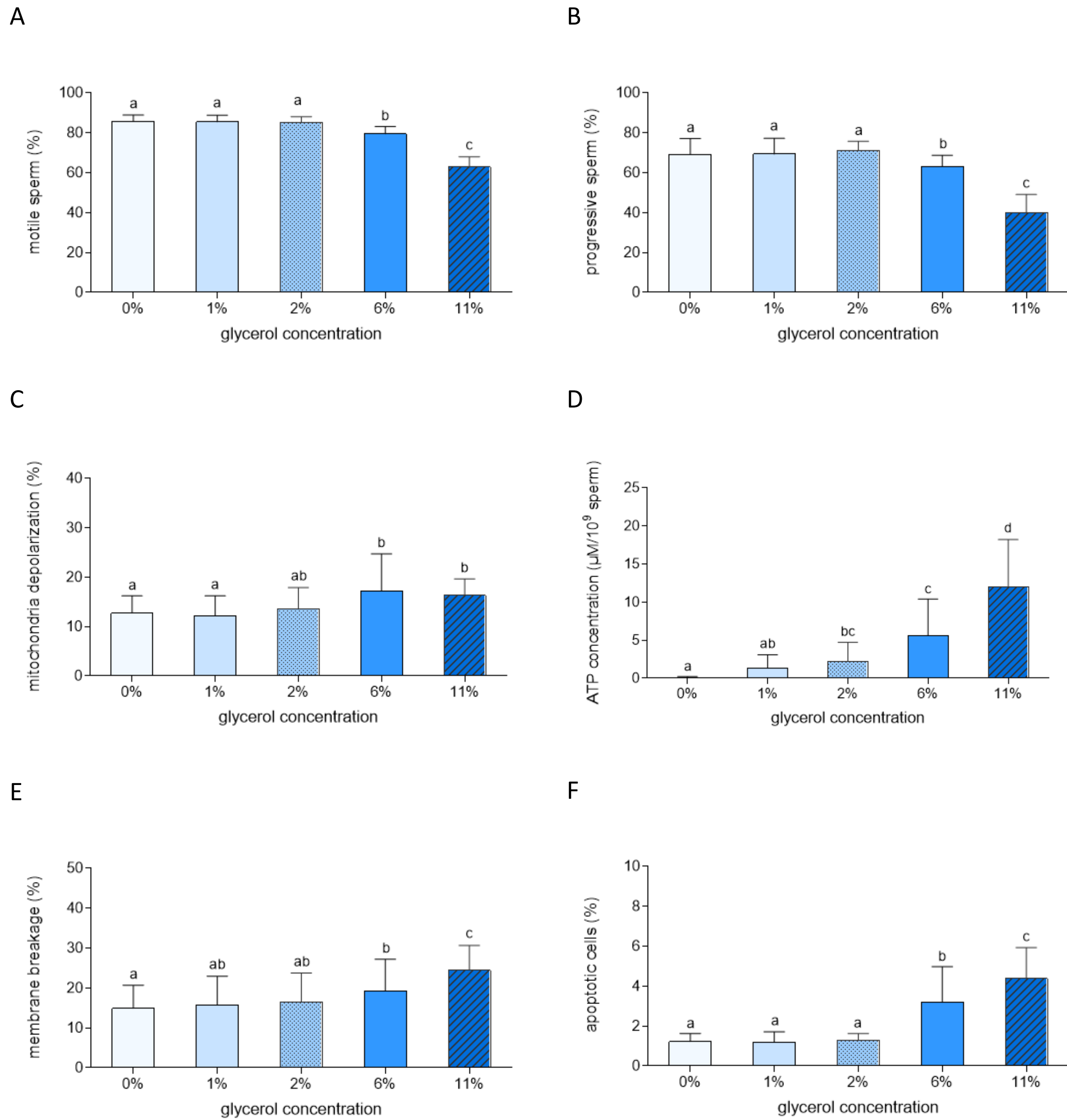
736 Abbreviation: GLY=glycerol concentration.



737

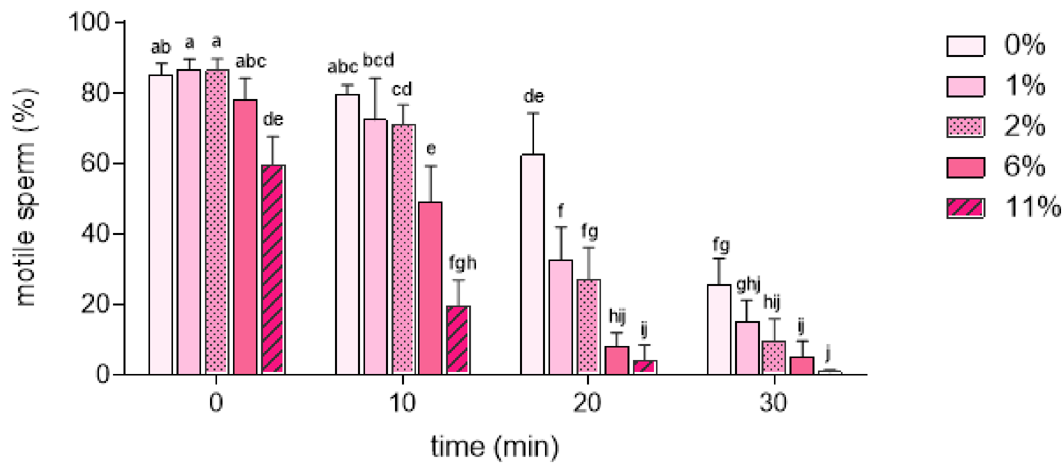
738 **Figure 4. Sperm penetration in perivitelline membrane (PVM) after pre-incubation with**
739 **different glycerol concentrations.** A and B represent the image (A) and the number of holes
740 (B) after PVM incubated with glycerolized sperm. Semen samples were collected and pooled
741 from 10 roosters and subjected to 0, 1, 2, 6 and 11% glycerol at 4 or 41°C for 10 min before
742 incubation with PVM (41°C for 20 min). The bars and lines correspond to the mean (5
743 experimental replicates) and the standard deviation of the mean. Different letters indicate
744 significant differences (p-value < 0.05).

745



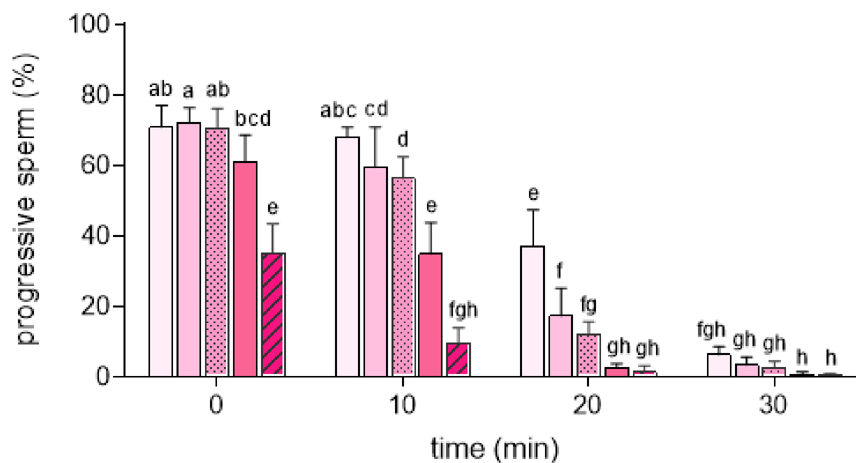
746 **Figure 5. Sperm quality parameters of glycerolized sperm at 4°C.** A-F represent evaluations
747 of different sperm quality parameters. Semen samples were collected and pooled from 10
748 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the
749 standard deviation of the mean. Different letters indicate significant differences (p-value <
750 0.05).
751

752 A



753

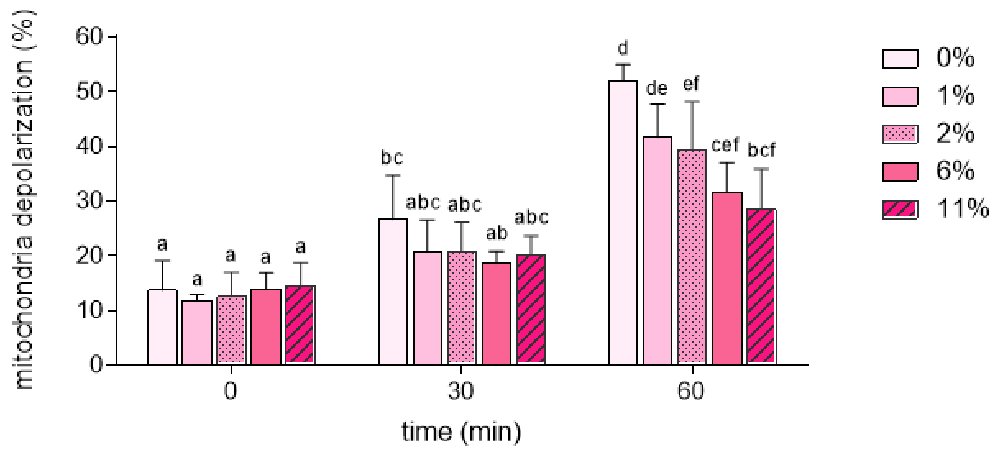
754 B



755

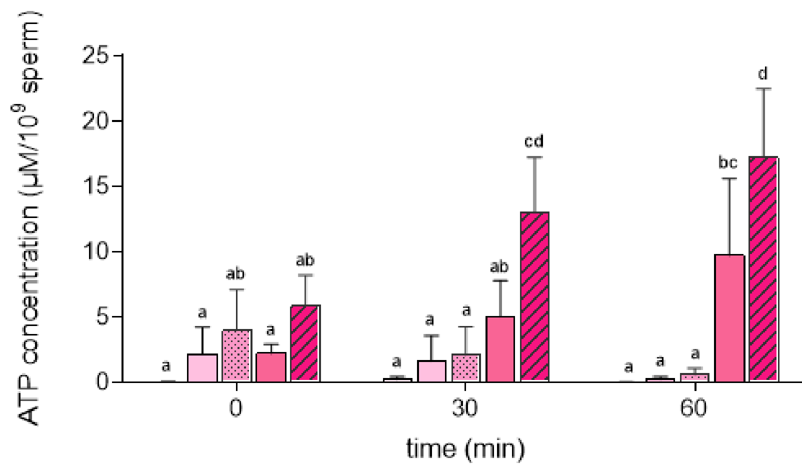
756 **Figure 6. Sperm motility of glycerolized sperm at 41°C.** A and B represent the percentages of
757 motile (A) and progressive sperm (B). Semen samples were collected and pooled from 10
758 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the
759 standard deviation of the mean. Different letters indicate significant differences (p-value <
760 0.05).
761

762 A



763

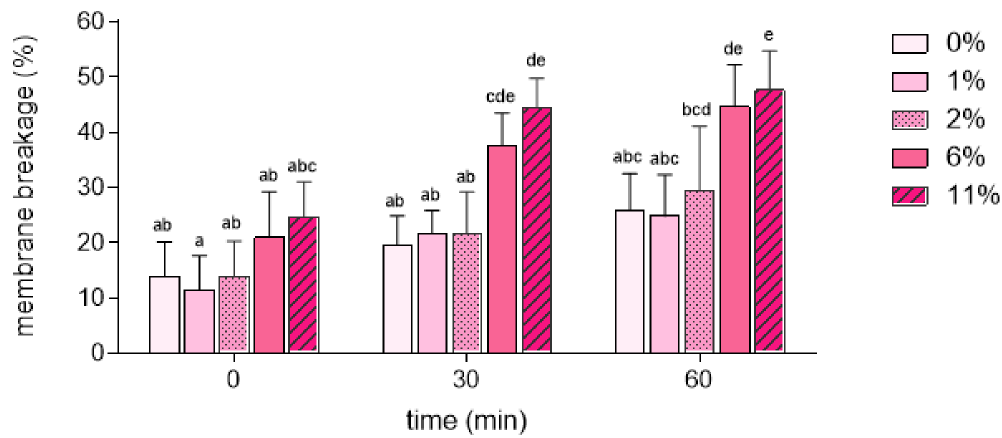
764 B



765

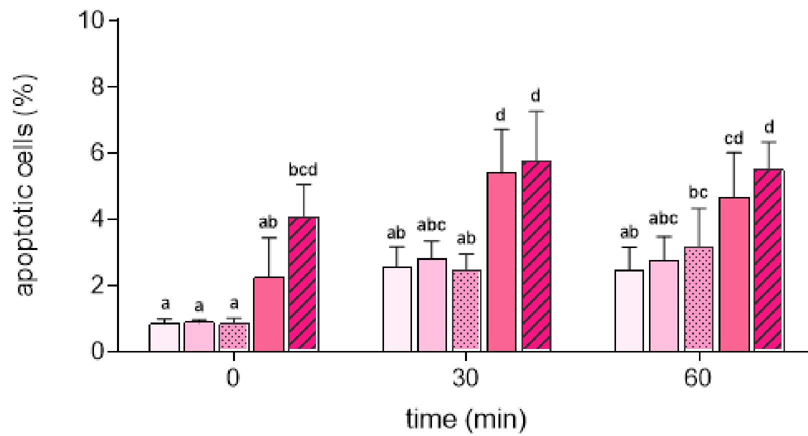
766 **Figure 7. Sperm mitochondria function and ATP concentration of glycerolized sperm at 41°C.**
767 A and B represent the percentages of mitochondria depolarization (A) and ATP concentration
768 (B). Semen samples were collected and pooled from 10 roosters. The bars and lines
769 correspond to the mean (5 experimental replicates) and the standard deviation of the mean.
770 Different letters indicate significant differences (p-value < 0.05).
771

772 A



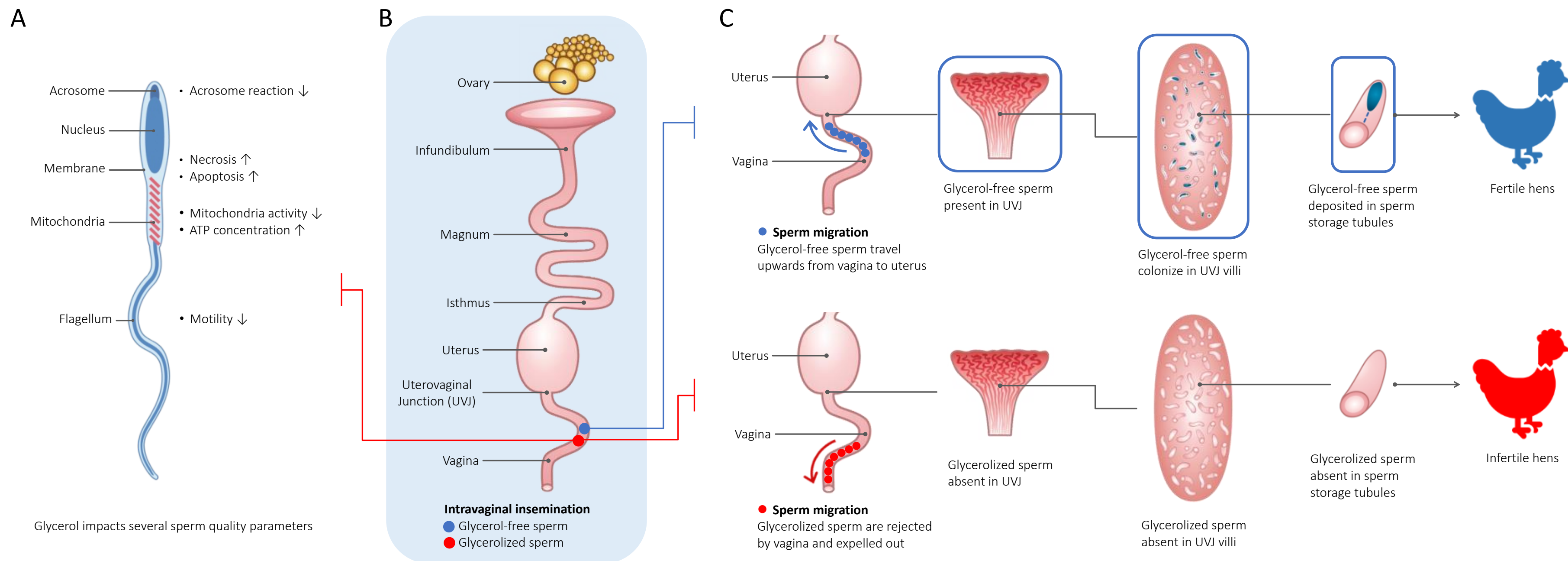
773

774 B



775

776 **Figure 8. Sperm membrane breakage and apoptosis of glycerolized sperm at 41°C.** A and B
777 represent the percentages of membrane breakage (A) and apoptosis (B). Semen samples were
778 collected and pooled from 10 roosters. The bars and lines correspond to the mean (5
779 experimental replicates) and the standard deviation of the mean. Different letters indicate
780 significant differences (p-value < 0.05).
781



782

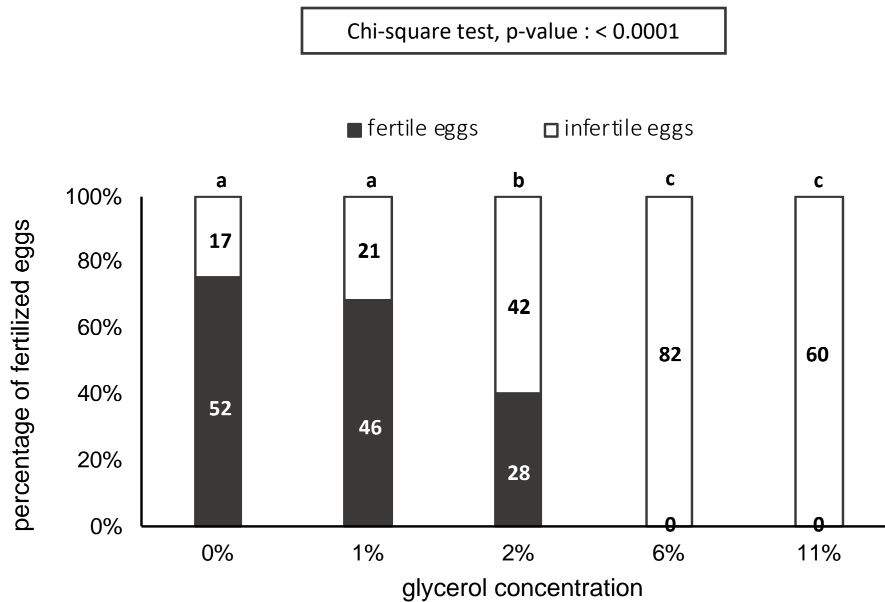
783 **Figure 9. Proposed model of glycerol impacts on sperm fertilizing capacity in chickens.** (A) indicates glycerol impacts on sperm biology:
784 increasing cell deaths and ATP contents, and decreasing acrosomal reaction, motility and mitochondria activity. (B) exhibits the intravaginal
785 insemination of glycerol-free or glycerolized sperm in hen's oviduct. (C) illustrates glycerol-free sperm deposited in sperm storage tubules of
786 uterovaginal junction, leading hens to produce fertile eggs. While glycerolized sperm are rejected and expelled by vagina, leading sperm absence
787 in sperm storage tubules and infertile hens.

788

789 **Supplementary Data**

790

791

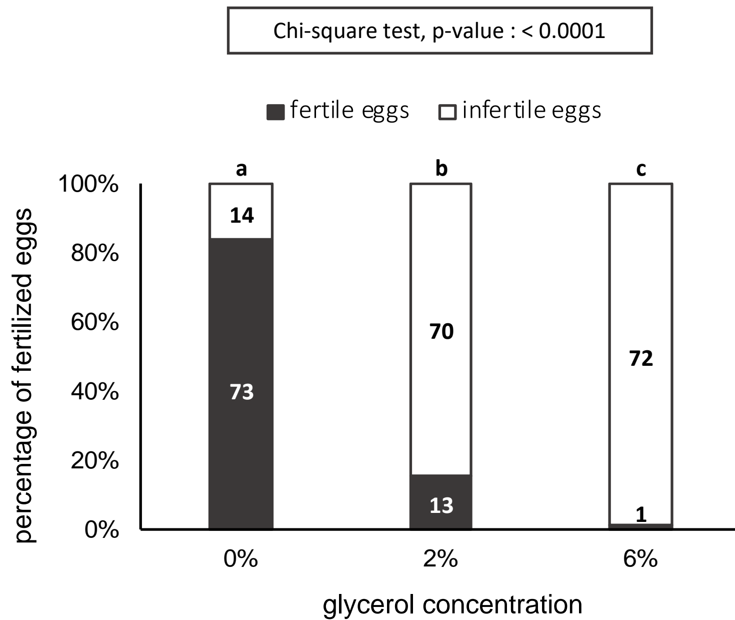


792

793 **Supplementary Data 1. Effect of glycerol presence in semen samples on sperm fertility**
794 **capacity.** Repeated experiment of Figure 1. Semen was collected and pooled from same
795 roosters but hens for each treatment were exchanged. Eggs were collected between 2nd and
796 7th day after first insemination in a dose of 100×10^6 sperm per hen for 2 consecutive days.
797 Black and white bars represent the percentage of fertile and infertile eggs. The numbers of
798 considered eggs are indicated on the bars. Different letters indicate significant differences (p-
799 value < 0.05).

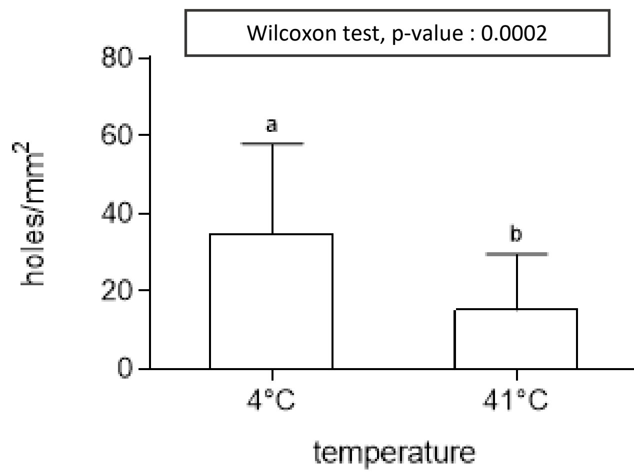
800

801



802

803 **Supplementary Data 2. Fertility of Hoechst 33342-stained glycerolized chicken sperm.**
804 Semen collected and pooled from 10 roosters. Number of hens=8 for each treatment (2
805 experimental replicates). Eggs were collected between 2nd and 7th day after insemination in a
806 dose of 200 × 10^{6} sperm per hen for 2 consecutive days. Black and white bars represent the
807 percentage of fertile and infertile eggs. The numbers of considered eggs are indicated on the
808 bars. Different letters indicate significant differences (p-value < 0.05).
809



810

811 **Supplementary Data 3. The number of holes after PVM incubated with glycerolized sperm.**
812 Semen samples were collected and pooled from 10 roosters and subjected to 0, 1, 2, 6 and
813 11% glycerol at 4 or 41°C for 10 min before incubation with PVM. Pieces of PVM were collected
814 individually from one egg for each experiment and then incubated with glycerolized sperm at
815 41°C for 20 min. The bars and lines correspond to the mean (5 experimental replicates) and
816 the standard deviation of the mean. Different letters indicate significant differences (p-value
817 < 0.05).
818

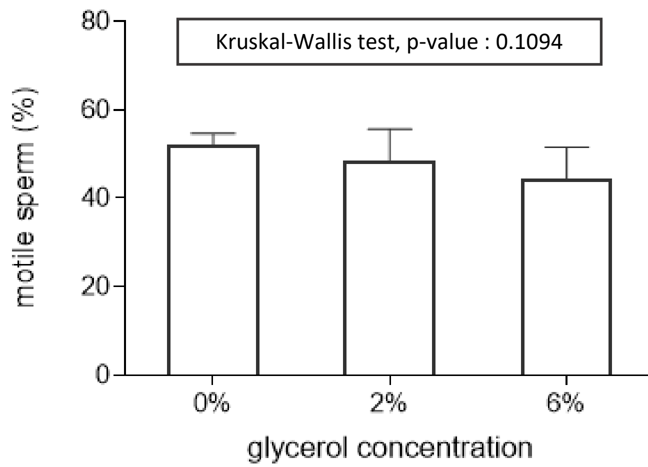
819 **Supplementary Data 4. Effect of glycerol concentration on *in vitro* sperm quality**
820 **parameters at 4°C**

	one-way ANOVA test (p-value)
motile sperm	**< 0.0001**
progressive sperm	**< 0.0001**
mitochondria activity	**0.0017**
ATP	**< 0.0001**
membrane breakage	**< 0.0001**
apoptosis	**< 0.0001**

821

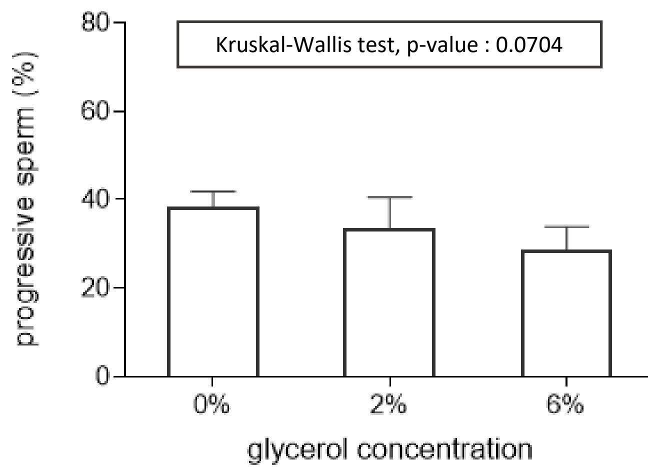
822

823 A



824

825 B



826

827 **Supplementary Data 5. Glycerolized sperm motility after *in vitro* incubation with sperm**
828 **storage tubules (SST) for 30 min.** A and B represent the percentages of motile (A) and
829 progressive sperm (B). The bars and lines correspond to the mean (5 experimental
830 replicates) and the standard deviation of the mean.